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Wood contains a cell-wall structural protein

(immunocytolocalization/loblolly pine/extensin/xylem/xylogenesis)

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ABSTRACT **A pine extensin-like protein (PELP) has been localized in metabolically active cells of differentiating xylem and in mature wood of loblolly pine (*Pinus taeda* L.). This proline-rich glycosylated protein was purified from cell walls of differentiating xylem by differential solubility and gel electrophoresis. Polyclonal rabbit antibodies were raised against the deglycosylated purified protein (dPELP) and purified antibody was used for immunolocalization. Immunogold and alkaline phosphatase secondary antibody staining both show antigen in secondary cell walls of earlywood and less staining in latewood. Immunoassays of milled dry wood were developed and used to show increased availability of antigen after hydrogen fluoride or cellulase treatment and decreased antigen after chlorite treatment. The specificity of the antigen–antibody reaction was confirmed by competition assays and by preadsorption of antibody to the purified protein. We propose that extensin-like protein is present in xylem cell walls during lignification and that the protein remains as a structural component of cell walls in wood for many years after xylogenesis. We suggest that such structural proteins play important roles in the differentiation of xylem and thereby could affect the properties of wood.**

Wood is a renewable natural resource that forms the basis of a large industry serving human needs for fiber and fuel. Wood is secondary xylem, formed by the vascular cambium of woody plants. The chemical composition and physical properties of wood have been the subject of intense research for the past century. Cellulose, lignin, hemicellulose, and extractives account for >99% of the mass of wood (1). Proteins have not been considered to be important components of wood.

Small amounts of nitrogen (by Kjeldahl analysis) can be detected in milled wood (2). Stable heartwood of pines contains nitrogen at ≈0.05% of the total dry weight (3). Amino acids can be recovered from wood by acid hydrolysis of extractive free wood (2). Laidlaw and Smith (2) found that amino acids accounted for at least 70% of the nitrogen in pine wood. They proposed that protein was the major nitrogenous material in wood but found that the amino acid composition of their material differed from that of Lamport and Northcote, who found a hydroxyproline-rich protein in cell walls of cultured cells of sycamore (*Acer pseudoplantanus*) (4). Cowling and Merrill (5) also suggested that extensin in the xylem primary cell walls could account for some of the nitrogen found in mature wood.

The proteins most likely to be found in wood are cell-wall structural proteins. Three kinds of cell-wall proteins, which differ in amino acid composition, have been found in plants (6). These are hydroxyproline-rich glycoproteins, glycine-rich proteins, and proline-rich proteins (6, 7). These proteins are generally considered to be important structural components of the plant cell walls. Localization studies of mRNA coding for cell-wall structural proteins and the localization of the proteins themselves (7–10) demonstrate tissue-specific expression in several different species of plants. Some structural proteins are associated with vascular systems (7, 9, 10). For example, proline-rich proteins are found by immunocytochemical localization to be associated with xylem vessel elements in soybean (7). Glycine-rich proteins in bean are located specifically in the tracheary elements of the protoxylem (9). However, there have been no reports of cell-wall structural proteins in wood cell walls (1).

Hydroxyproline, the major amino acid of extensin, has been measured in cell walls of cultured cells from *Gingko biloba*, *Cupressus* sp., and *Ephedra* sp., suggesting the presence of extensin-like proteins in gymnosperms (11). Hydroxyproline was also found in low amounts (about 5% of the total amino acids) in acid hydrolysates of wood from *Pinus sylvestris* (2). In this paper, an extensin-like cell-wall protein of loblolly pine (*Pinus taeda*) is identified and purified. Immunolocalization shows that the protein is present in cell walls of mature wood.

MATERIALS AND METHODS

Plant Materials. All plant materials were obtained from loblolly pine growing in the North Carolina coastal plain. Differentiating xylem was collected in May or June from rapidly growing trees 11–13 years old. Bark can be peeled from the stems of trees during this time. The differentiating xylem remained attached to the wood, while the cambium and phloem stayed attached to the bark. The differentiating xylem was removed with vegetable peelers and was frozen immediately in liquid nitrogen. The stored material was used as a source of cell-wall proteins during the rest of the year.

Fresh wood of 4- to 6-year-old loblolly pine was cut with a sledge microtome into 20-μm sections for immunolocalization. Pieces of dried wood from 10-year-old stems (dried for 2 years at room temperature) were milled into powder for immunoassays.

Purification and Analysis of the Cell-Wall Structural Protein. Frozen loblolly pine xylem was ground to a powder in a blender in liquid nitrogen and suspended in extraction buffer (0.1% potassium acetate, pH 5.0/4 mM $Na_2S_2O_5$). The cell-wall fraction was obtained by filtration and washing seven times in 2 mM $Na_2S_2O_5$. A protein fraction was extracted from the cell walls with 0.4 M calcium chloride (8) and was further fractionated by differential solubility in 5% CCl_3COOH (12). The resulting cell-wall protein was further purified by SDS/PAGE (7.5% polyacrylamide) (13) and electroelution (14). The protein, called pine extensin-like protein (PELP), was deglycosylated (dPELP) by hydrogen fluoride (15). Amino acid analysis by phenyl isothiocyanate derivatization was done as described (16) after acid hydrolysis (17). The hydrolyzed amino acids were separated by reverse-

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Abbreviations: PELP, pine extensin-like protein; dPELP, deglycosylated PELP; FPLC, fast-protein liquid chromatography.

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phase C_{18} HPLC (16) and identified by comparison to amino acid standards (Sigma).

Immunological Assays. Purified dPELP was used as antigen to inject two New Zealand White rabbits. Bands containing the dPELP were cut from gels (SDS/PAGE), dried under vacuum (Speedvac), and ground to a fine powder. The protein (50 μg) was mixed with complete Freund's adjuvant for the primary injection. A second injection was carried out 45 days later with 30 μg of dPELP mixed with incomplete adjuvant. Serum was collected 2 weeks later for ELISA and immunoblotting (Western blotting) (18). ELISA was used to titer the serum and to estimate the relative amounts of antigen. Preimmune serum was used routinely for controls. Alkaline phosphatase-conjugated goat anti-rabbit serum (Sigma) was used as the secondary antibody. Proteins for Western blots were separated on SDS/PAGE (7.5% polyacrylamide) and blotted onto nitrocellulose. Tris-buffered saline (TBS) used for immunoassays was 50 mM Tris·HCl, pH 7.6/0.15 M NaCl.

Purification of Anti-dPELP IgG. Rabbit anti-dPELP serum was purified by ion-exchange chromatography (Mono Q, Pharmacia) on a fast protein liquid chromatography (FPLC) column [0.0–0.3 M NaCl gradient in 20 mM Tris·HCl (pH 8.0)]. The relative amount of IgG in different fractions was determined by ELISA with goat anti-rabbit IgG coupled to alkaline phosphatase. Anti-dPELP activity was also determined by ELISA plate assays. Further purification of the anti-dPELP antibodies was carried out by affinity purification with dPELP antigen immobilized on nitrocellulose (19). Briefly, a specific band of purified dPELP electrophoresed on SDS/PAGE and blotted onto nitrocellulose was cut out and incubated with the anti-dPELP active fraction eluted from the Mono-Q column. This specific antibody fraction was eluted from the membrane with 0.2 M glycine (pH 2.8) and used for immunolocalization on wood sections.

Immunolocalizations. For paraffin embedding, fresh tissue segments were fixed in 4% paraformaldehyde/0.25% glutaraldehyde/100 mM potassium phosphate (pH 7.0), and 8-μm sections were cut on a Leitz 1512 microtome. Cellulase treatment of sections used 100 units of enzyme per ml in 0.1 M sodium acetate (pH 5.0). For immunolocalization of antigen in wood, pieces and sections were blocked with 3% bovine serum albumin in TBS for 30 min and incubated with 1:2000-diluted preimmune serum or purified immune serum (by specific binding) in blocking solution for 2 hr. 5-Bromo-4-chloro-3-indolyl phosphate was used with nitro blue tetrazolium for alkaline phosphatase staining. Colloidal gold-conjugated goat anti-rabbit serum (Sigma) was used with silver enhancement by following the manufacturer's instruction (Sigma).

Milled Wood Immunoassay. Antibody was used to demonstrate the presence of PELP in samples of milled wood. The milled wood was washed in water and blocked with 3% bovine serum albumin in TBS. The blocked milled wood was incubated with rabbit anti-dPELP, washed in TBS containing 0.05% Tween, and then incubated with the secondary antibody. The resulting complex was incubated with *p*-nitrophenyl phosphate as substrate at room temperature. The milled wood settled out, and the absorbance at 405 nm of the supernatant was determined.

Treatment of Milled Wood. Milled wood was treated with cellulase as described earlier for wood sections to make PELP in wood more available for antibody binding. Milled wood was also treated with hydrofluoric acid to hydrolyze polysaccharides from wood. One gram of milled wood was incubated for 1 hr with 5 ml of 70% hydrofluoric acid in pyridine and then washed with water several times. Some milled wood was treated with acidified sodium chlorite (0.6% sodium chlorite acidified with acetic acid to pH 3.5) at 65°C for 1 hr, which dissolves lignin and cross-linked protein in wood (20, 21).

RESULTS

Protein Purification. A PELP fraction was isolated from differentiating xylem of loblolly pine. Cell walls were obtained from 200 to 400 g of loblolly pine xylem as described, and the cell-wall fraction was extracted with 0.5 M $CaCl_2$. The extracted protein was then precipitated with 5% CCl_3COOH to obtain a CCl_3COOH-soluble fraction. This fraction showed a single band on SDS/PAGE (Fig. 1*a*) with low mobility. A band of the same mobility as PELP was visible with crude cell-wall extracts of differentiating xylem (Fig. 1*a*, lane 3). This result suggests that PELP is an abundant component of the total cell-wall protein. When using the whole-cell soluble extract or total cell-wall proteins, a band was visible in the same position on a Western blot prepared with PELP antibodies (Fig. 1*b*, lanes 1 and 3). The final CCl_3COOH-soluble fraction was about 1/400th of the total soluble protein and about 1/6th of the cell-wall $CaCl_2$-extracted protein.

Properties of the PELP Fraction. Treatment of PELP with hydrofluoric acid in pyridine increased the SDS/PAGE mobility of the protein, indicating that the protein is glycosylated (Fig. 1*a*, lane 7) and has an apparent molecular mass of 68 kDa. We hydrolyzed the dPELP fraction with 6 M HCl and determined the relative proportion of amino acids (Table 1). The protein is rich in proline (25%) and hydroxyproline (11%), consistent with a structural role.

Properties of Anti-dPELP Antibody. Antibodies against dPELP were obtained from New Zealand White rabbits. Binding in ELISA to 10 ng of antigen was observed with antibody dilutions as high as 1:512,000. The antibodies did not bind to bovine serum albumin, and no cross reaction was detected with preimmune serum at 1:500 dilution. The dPELP antibodies also bound to glycoprotein PELP, but at a lower dilution (1:64,000). Western blots of purified protein showed staining for both glycosylated and deglycosylated forms of the protein (Fig. 1*b*). Whole-cell soluble extract also showed antigen both before and after deglycosylation. Preimmune serum did not bind to the antigen on control blots. Some minor bands were seen at lower molecular masses in the crude extract after deglycosylation (Fig. 1*b*, lane 2).

Further Purification of Antiserum and Specificity of Antibodies. Antiserum against the cell-wall structural protein was purified by FPLC using ion-exchange chromatography on a

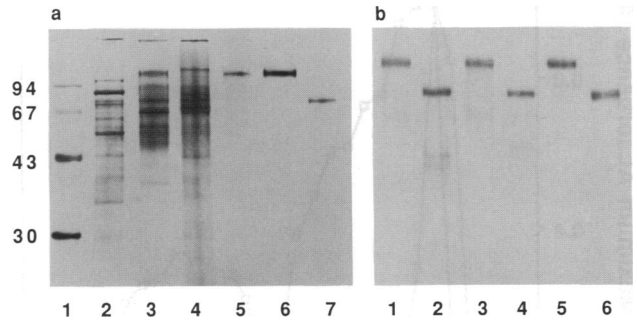

FIG. 1. Characterization of PELP by SDS/PAGE (*a*) and by immunoblotting (*b*). (*a*) Lanes: 1, marker proteins; 2, crude xylem cell extract; 3, cell-wall salt extract; 4, CCl_3COOH-precipitated proteins of cell-wall salt extract; 5, CCl_3COOH supernatant of cell-wall salt extract; 6, purified PELP; 7, purified dPELP. (*b*) Lanes: 1, crude extract of differentiating xylem; 2, deglycosylated crude extract; 3, cell-wall salt extract; 4, deglycosylated cell-wall salt extract; 5, purified PELP; 6, purified dPELP. One microgram of protein was used in each lane except lane 1 in *b* (5 μg) and lanes 7 and 6 in *a* (0.2 μg).

Table 1. Amino acid analysis of PELP

Residue	Mol %
Aspartic acid	8.06
Glutamic acid	3.24
Hydroxyproline	11.47
Serine	6.34
Glycine	7.86
Histidine	0.78
Threonine	4.82
Arginine	2.19
Alanine	6.44
Proline	24.04
Tyrosine	4.06
Valine	5.66
Methionine	0.75
Cysteine	ND
Isoleucine	1.34
Leucine	2.97
Tryptophan	ND
Phenylalanine	1.52
Lysine	8.46

Mol % values were from amino acid analysis of 6 M HCl hydrolysates. The amounts of cysteine and tryptophan were not determined (ND).

Mono-Q column (Pharmacia). The elution profiles of IgG and anti-dPELP activity, eluted from the column by a salt gradient, were distinct (Fig. 2). Anti-dPELP activity was found in only a few fractions. Specificity of purified anti-dPELP serum binding to milled wood was further tested by competition experiments. PELP protein could competitively block the binding of antibody to milled wood (Fig. 3), whereas bovine serum albumin and lignin did not compete. Similarly, cellulose and hemicellulose did not competitively block antibody binding even at milligram levels. Extractive-free milled wood showed binding of anti-dPELP antibody at levels comparable to that of untreated milled wood; therefore, extractives are unlikely to be involved in antibody binding. In additional experiments, dPELP antibody was preadsorbed to nitrocellulose filters containing purified PELP protein. The "depleted" antibody was subsequently tested for binding to milled wood and assayed for activity. "Depleted" antibody was much less effective. We conclude that the antibody

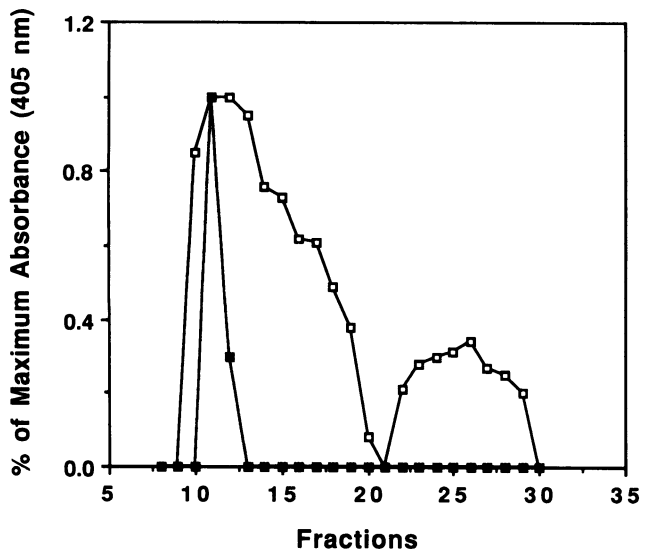

FIG. 2. Purification of anti-dPELP antibody. ELISA determinations of IgG and anti-dPELP antibodies analyzed by Mono-Q FPLC ion-exchange chromatography. ■, Anti-dPELP activity; □, distribution of total IgG.

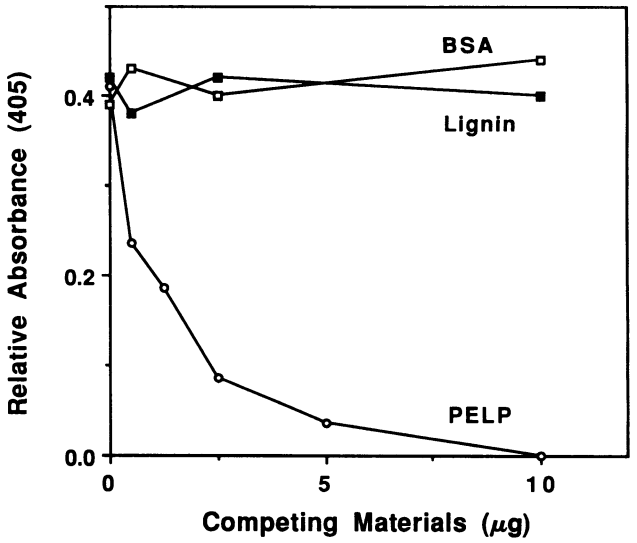

FIG. 3. Immunoassay competition of cell-wall components. Different components were tested for their ability to compete for antibody binding with the antigen in the milled wood.

binding to dPELP shows negligible affinity for any other components of the cell wall and that antibody reacts with high relative specificity to the PELP component of the cell wall.

PELP Remains in Wood That Is Cut and Dried. We have assayed samples of dried wood from loblolly pine for PELP antigenicity. Material was obtained from 11-year-old trees cut 2 years earlier and dried at room temperature. Antigen was detected in an internal section of 6-year-old wood, ground in a Wiley mill. A spectrophotometric assay of conjugated enzyme activity showed that antigen remained associated with the ground wood (Fig. 4). As expected, increased levels of antigen could be observed after hydrolysis by hydrofluoric acid or enzymatic digestion by cellulase (Fig. 4). Presumably, treatment with cellulase or deglycosylation by hydrogen fluoride results in greater availability of the antigen. After treatment with sodium chlorite, which reduces lignin (20) and aromatic amino acids (21), the availability of antigen decreased. We conclude that PELP is imbedded in

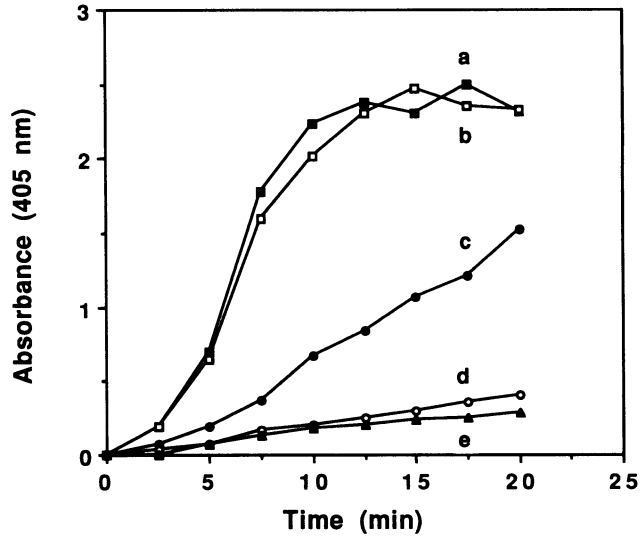

FIG. 4. Immunoassay of treated milled wood. Milled wood samples were treated with hydrofluoric acid [sample a (■)], cellulase [sample b (□)], and acidified sodium chloride [sample d (○)]. Sample c (●) was untreated, and sample e (▲) was assayed with preimmune serum instead of anti-PELP antibody.

the wall and is stable for years after the synthesis and differentiation of wood.

Immunolocalization of PELP in Wood. Sections of fresh wood samples cut by hand or with the sledge microtome were tested with dPELP antibody for antigen localization. Antigen was readily apparent in metabolically active tissue (Fig. 5 *a* and *b*). On further examination, differential staining of mature xylem (wood) compared with that of preimmune serum controls was observed (Fig. 5 *c* and *d*). The antigen was widely distributed in wood. The antigen was more evident in rays (Fig. 5*a*) and was more apparent in fractured tracheids than on an exposed surface split between tracheids (Fig. 5*c*). Antibody staining increased after wood sections were pretreated with cellulase. Significant amounts of cell-wall extensin-like protein appeared to be present in mature wood. Similar results were obtained with heartwood. When serum was preadsorbed with antigen immobilized on nitrocellulose, specific staining was not observed (data not shown).

PELP Antigen Is Present in the Secondary Wall. In sections examined under higher magnification after treatment with cellulase, antigen was seen in the layers of the secondary wall (Fig. 5 *e–j*). Staining was observed by using immunogold-conjugated secondary antibody (Fig. 5 *g* and *i*) and with alkaline phosphatase (Fig. 5 *e* and *j*). Staining was reduced in the middle lamellae and was present in different layers of the secondary wall. Some preferential labeling was associated with bordered pits (Fig. 5*i*). Latewood appeared to be poorly labeled by antibody (Fig. 5*j*).

DISCUSSION

We conclude that PELP is an extensin-like cell-wall structural protein because of its location, abundance, solubility, glycosylation, and amino acid composition. PELP is different from several other extensin proteins that have a higher content of hydroxyproline (11). PELP is also found in the xylem secondary cell wall, and significant amounts remain years after the maturation of wood. To some extent, our results were anticipated by Laidlaw and Smith (2), who looked for extensin in wood of Scots pine in 1965. They did not conclude that extensin was present in wood, presumably because the hydroxyproline content was low. Only about 5% of their recovered amino acids were accounted for by hy-

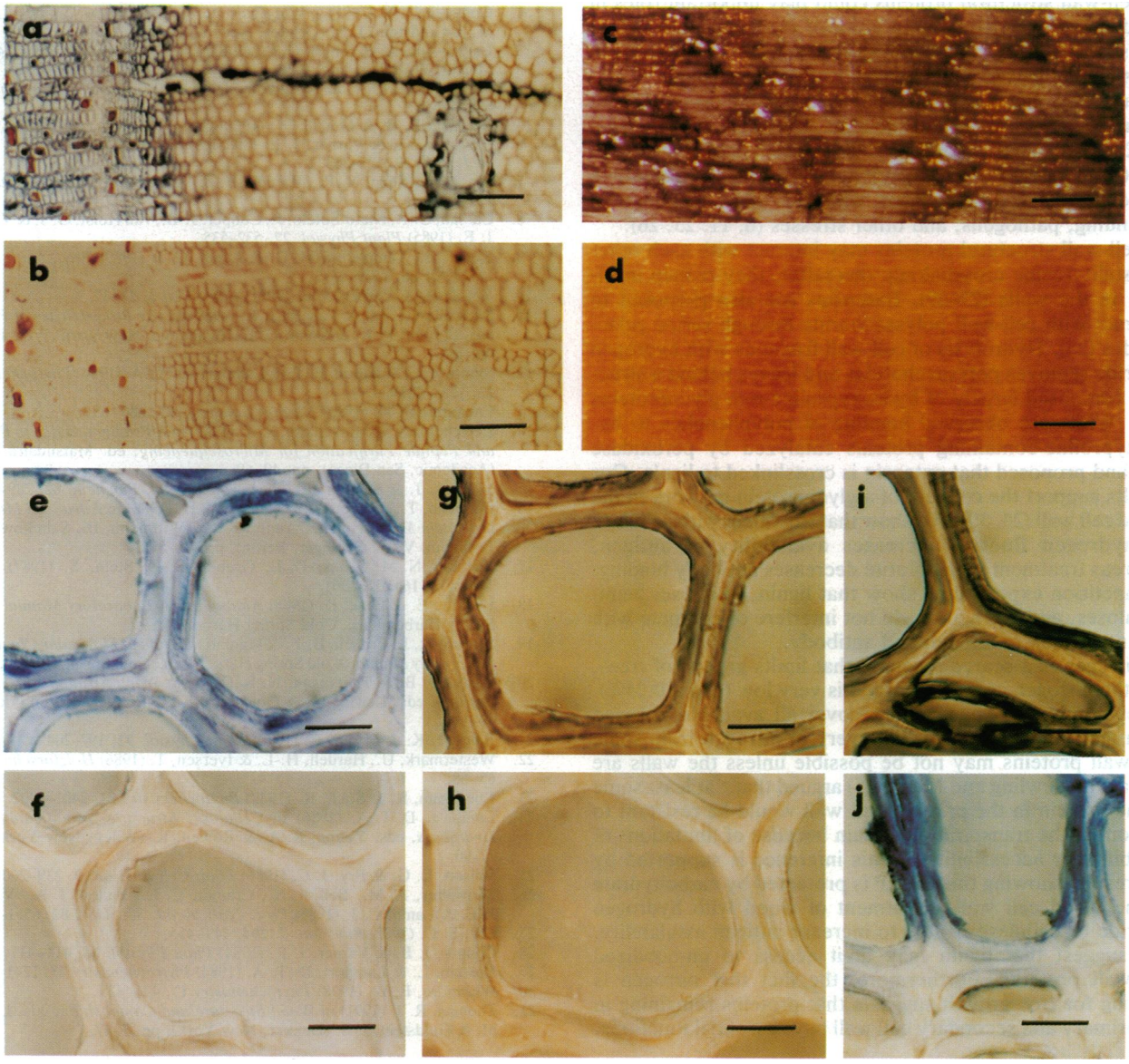

FIG. 5. Immunolocalization of PELP in wood and young stem sections. Alkaline phosphatase-conjugated (*a–f* and *j*) or colloidal gold-conjugated (*g–i*) goat anti-rabbit serum was used as the secondary antibody. Paraffin-embedded cross sections of young stems (*a* and *b*), mature wood longitudinal pieces (*c* and *d*), and mature wood cross sections (*e–j*) were treated with either anti-dPELP (*a*, *c*, *e*, *g*, *i* and *j*) or preimmune serum (*b*, *d*, *f* and *h*). (Bars = 100 μm in *a* and *b*, 200 μm in *c* and *d*, and 10 μm in *e–j*.)

droxyproline in contrast with results of Lamport and Northcote (4), who found that hydroxyproline accounted for about 13% of amino acid composition of cell walls from sycamore-cultured cells. In combination with our results, their results now suggest that more than one protein is present in wood and that the undefined proteins are not rich in proline/hydroxyproline.

Further support for presence of extensin in the primary wall of spruce (*Picea abies*) comes from the work of Westermark *et al.* (22), who analyzed wood samples enriched for the compound middle lamella. They found nitrogen in fractions of the middle lamella and inferred that a protein component of the primary wall was retained in the lignified wood. Further work is needed to identify other proteins that may be present in mature wood and to ascertain their origin. Ebermann and Stich (23) reported activity for peroxidase in sapwood and heartwood of three hardwoods (*Acer campestris, Acer plantoidies*, and *Quercus robur*) and four softwoods (*Pinus nigra austriaca, P. sylvestris, Taxus baccata*, and *Picea excelsa*). In sapwood from *Ailanthus* sp., amylase activity was detected (23).

Cell-wall structural proteins could play important roles in the development and differentiation of xylem and could affect the structure and composition of wood. Extensin-like proteins could provide a mechanical role in the secondary wall, or, alternatively, they could play a role in the establishment of the structural pattern of the other components of the wall (6, 24). Both proline-rich and glycine-rich proteins are deposited in the wall before lignification (7). Some extensin-like proteins are also associated with the plant response to wounding, pathogens, and other stresses (6, 11, 25, 26).

Cell-wall structural proteins may interact with each other by isodityrosine crosslinking; thereafter, they remain insoluble in the walls (27–29). Extensins are also proposed to interact with cell-wall polysaccharide through serine galactosides (25). Hydrolysis of cell-wall fractions with hydrogen fluoride removes polysaccharides and leaves behind a structure composed largely of hydroxyproline-rich glycoproteins (15). Whitmore observed bonding of polyphenolics to hydroxyproline-containing proteins catalyzed by peroxidase (30) and proposed that extensin is crosslinked to lignin. Our results support the concept of a glycoprotein network in the wood cell wall (25, 30). We show that treatment with cellulase or hydrogen fluoride increases availability of antigen, whereas treatment with chlorite decreases antibody binding. Competition experiments show that lignin, cellulose, hemicelluloses, and extractives do not interfere or compete with the interaction of antigen and antibody.

Nitrogen is a scarce resource that limits growth of trees, and the nitrogen content of wood is very low (about 0.05%). Trees would be expected to recover all available nitrogen from mature xylem (3, 5). Recovery of nitrogen from some cell-wall proteins may not be possible unless the walls are degraded. Cowling and Merrill (5) argued that "at least some of the protein in the primary cell wall would be resistant to elution by the transpiration stream because of limitations of solubility or accessibility." This inference is supported by our results showing that PELP is protected by carbohydrate in the wood cell wall. Treatment of wood with hydrogen fluoride or cellulase is able to increase antigen availability. We suggest that lignin may limit access to immobilized cell-wall proteins. Furthermore, the fact that nitrogen is limiting for tree growth suggests that proteins remaining in the primary and secondary cell wall have essential functions.

A further question concerns the preferential location of antigen in earlywood compared to latewood. Earlywood of white pine was found to contain more nitrogen than latewood by a ratio of 1.17 (3). Many aspects of earlywood and latewood are different (31)—e.g., latewood has thicker walls and fewer pits. The relationship of cell-wall proteins in reaction wood formation is another question of interest because cell-wall proteins are known to be involved in the wounding response, which may be related to the response to mechanical stress.

Further study of the pine extensin-like genes and proteins in formation of wood should lead to a deeper understanding of cell-wall structure and wood properties. The potential roles of cell-wall structural proteins should now be considered in models of secondary cell-wall formation and xylogenesis. Also, the presence of protein in wood may have implications for the processing of wood and paper.

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